

Structures and cytotoxicities of three new sesquiterpenes from cultures of *Armillaria* sp.

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Abstract: Three new sesquiterpene aryl esters, named 10-dehydroxy-melleoliede B (**1**), 1-*O*-formyl-10-dehydroxy-melleoliede B (**2**) and 10-oxo-melleoliede B (**3**) together with six known ones (**4**–**9**), were isolated from the cultures of *Armillaria* sp. The structures of the new compounds were elucidated based on the extensive spectroscopic methods. Compounds **1**, **2**, and **5**–**9** exhibited moderate cytotoxicities.

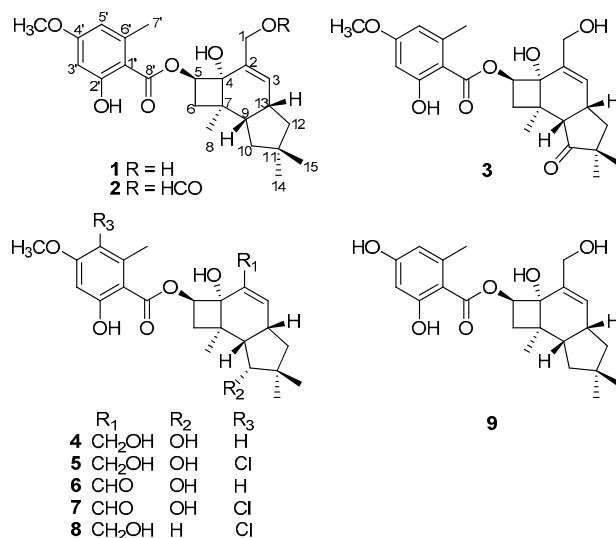
Keywords: basidiomycete, *Armillaria* sp., sesquiterpene aryl ester, cytotoxicities

Introduction

The basidiomycete genus *Armillaria* is recognized for the production of rich secondary metabolites with structure diversities as well as interesting biological activities.¹ Among those metabolites, sesquiterpene aryl esters are quite a big family which showed antibacterial, antifungal activities,^{1c,i} and cytotoxicity.^{1o,1p} As a part of our search for naturally occurring secondary metabolites with diverse structures from fungi in China,² we have carried out the chemical investigation of *Armillaria* sp. cultures, which led to the isolation of three new sesquiterpene aryl esters, named 10-dehydroxy-melleoliede B (**1**), 1-*O*-formyl-10-dehydroxy-melleoliede B (**2**), and 10-oxo-melleoliede B (**3**), together with melleoliede B (**4**),^{1g} 5'-chloromelleoliede (**5**),^{1g} armillarigin (**6**),^{1g} armillarikin (**7**),^{1g} A52a (**8**),³ and armillane (**9**).^{1p} Those new compounds were elucidated by means of spectroscopic methods, while the known compounds were identified by comparison with data in the literature. All of these compounds were evaluated for cytotoxicity against five human cancer cell lines.

Results and Discussion

Compound **1**, obtained as a colorless oil, had the molecular formula of C₂₄H₃₂O₆ based on the negative-ion HRESIMS at *m/z* 415.2118 [M – H][–] (calcd for C₂₄H₃₁O₆, 415.2120). The IR data exhibited absorption bands for a hydroxy at 3430 cm^{–1}, an ester group at 1646 cm^{–1} and a substituted aromatic ring at 1617 cm^{–1} and 1578 cm^{–1}, which consisted with the UV spectra. The ¹H NMR spectrum (Table 1) of **1** exhibited one



chelated phenolic hydroxy proton at δ_H 11.63, two aromatic protons at δ_H 6.31, one methoxy group at δ_H 3.80, four methyls including an aromatic one at δ_H 2.41. The ¹³C NMR spectrum revealed 24 carbons resonances (Table 2), which were classified into five methyl groups (three aliphatic, one oxygenated and one aromatic), four aliphatic methylenes (one oxygenated), six methines (three olefinic), and nine quaternary carbons (one carbonyl), indicating the presence of a tetra-substituted benzene unit as well as one ester carbonyl signal at δ_C 171.8. The above data were quite closely related to those of melleoliede B (**4**).^{1g} The significant differences of the ¹³C NMR data between **1** and **4** were the lack of a secondary alcohol

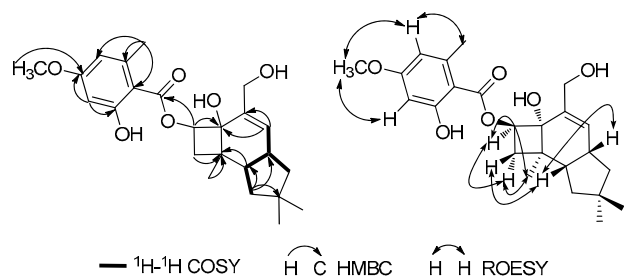
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Table 1. ^1H NMR data of **1–3** (**1, 3** in $\text{Me}_2\text{CO}-d_6$; **2** in CDCl_3)

No.	1 δ_{H} (J in Hz)	2 δ_{H} (J in Hz)	3 δ_{H} (J in Hz)
1	4.31, br. d (12.3); 4.03, dd (12.3, 2.9)	4.72, d (12.3); 4.60, d (12.3)	4.29, br. d (13.6); 4.00, br. d (13.6)
3	5.77, br. s	5.90, br. s	5.98, br. s
5	5.64, t (8.7)	5.48, t (8.7)	5.67, t (8.6)
6	1.94, dd (11.2, 8.7); 1.70, dd (11.2, 8.7)	1.97, dd (11.1, 8.7); 1.77, dd (11.1, 8.7)	1.95, dd (11.3, 8.7); 1.73, dd (11.3, 8.7)
8	1.25, s	1.28, s	1.64, s
9	2.19, m	2.19, m	2.64, d (7.5)
10	1.45, overlapped; 1.38, overlapped	1.44, overlapped; 1.24, overlapped	
12	1.86, dd (13.2, 8.5); 1.50, overlapped	1.85, dd (13.4, 8.7); 1.48, overlapped	2.09, overlapped; 1.91, br. d (13.0)
13	2.78, br. t (7.8)	2.78, br. t (7.7)	3.13, br. t (7.5)
14	1.00, s	0.95, s	0.99, s
15	1.00, s	0.99, s	0.97, s
3'	6.31, br. s	6.32, d (2.4)	6.31, br. s
5'	6.31, br. s	6.26, d (2.4)	6.32, br. s
7'	2.41, s	2.40, s	2.43, s
OCH ₃	3.80, s	3.79, s	3.81, s
Ar-OH	11.63, s	11.61, s	11.60, br. s
CHO		7.87, s	

signal and the presence of a methylene signal at δ_{C} 41.9 in **1**, revealing the absence of HO-10 in **1**. The above assumption was further confirmed by the ^1H - ^1H COSY correlations of H-10/H-9/H-13/H-12, and the HMBC correlations from H-10 to C-9, C-11, and C-13 (Figure 1).

The ROESY correlations (Figure 1) of H-9/H-13, H-9/H-6 β , H-5/H-6 α , H-5/H-8, and H-6 α /H-8 of **1**, as well as the similar chemical shifts of the chiral carbons comparing with those of melleolide B, suggested the *cis-anti-cis* stereochemistry of the three rings.⁴ Therefore, the structure of compound **1** was established as 10-dehydroxy-melleolide B.

**Figure 1.** Key HMBC, ^1H - ^1H COSY, and ROESY correlations for **1**

Compound **2**, purified as a colorless oil, had a molecular formula of $\text{C}_{25}\text{H}_{32}\text{O}_7$ according to its negative-ion HRESIMS at m/z 443.2070 ($[\text{M} - \text{H}]^-$, calcd, 443.2069). The IR spectrum showed the presence of a hydroxy group at 3432 cm^{-1} and an ester moiety at 1629 cm^{-1} . The 1D NMR (Tables 1 and 2) data were quite similar with those of **1** except for an additional signal for a formyl group (δ_{H} 7.87, δ_{C} 160.8). The formyl group attached at C-1 was confirmed by the HMBC of H-1 to C-CHO. Detailed analysis of 1D and 2D NMR data suggested that the other parts of **2** were the same to those of **1**. Therefore, compound **2** was established as 1-O-formyl-10-dehydroxy-melleolide B.

Compound **3**, was obtained as a colorless oil. The negative-ion HRESIMS afforded the molecular formula of $\text{C}_{24}\text{H}_{30}\text{O}_7$ at m/z 429.1917 ($[\text{M} - \text{H}]^-$, calcd, 429.1913). The IR data exhibited absorption bands for a hydroxy at 3431 cm^{-1} , an ester group at 1642 cm^{-1} and a substituted aromatic ring at 1618 cm^{-1} and 1579 cm^{-1} , which were similar with those of compound **1**. Detailed analysis of the ^{13}C NMR suggested that the main difference between **3** and **1** was that a methine was oxidized into a keto carbonyl group at δ_{C} 220.6 (C-10), which resulted in the downfield shift of C-9 ($\Delta\delta$ 7.1 ppm), C-11 ($\Delta\delta$ 6.2 ppm) in compound **3**. The above conclusion was supported by the HMBC correlations of H-9, H-13, and H-14 to C-10, and H-3 (δ_{H} 5.98) to C-12 (δ_{C} 41.9). Further analysis of 1D and 2D NMR data and coupling constants suggested that the other parts of the molecular was the same to compound **1**. Thus, compound **3** was established as 10-oxo-melleolide B.

All compounds were evaluated for their cytotoxicity against five human cancer cell lines using the MTT method as reported previously.⁵ Compounds **1**, **2**, and **5–9** showed cytotoxicities as shown in Table 3. It is notable that compound **2** showed stronger cytotoxicities than that of the positive control in MCF-7 and SW480 cell lines.

Table 3. Cytotoxicity for compounds **1–9** (IC_{50} , μM)

Entry	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	17.79	20.90	16.79	16.49	17.44
2	14.50	23.16	18.41	5.34	10.77
3	> 40	> 40	> 40	> 40	> 40
4	> 40	> 40	> 40	> 40	> 40
5	> 40	> 40	20.11	30.06	> 40
6	14.27	19.51	10.01	10.67	19.19
7	14.11	17.41	14.73	11.52	19.89
8	17.06	17.77	15.89	14.10	15.70
9	20.27	30.36	16.62	16.56	> 40
cisplatin ^a	1.3	15.4	17.3	15.8	24.4

^aCisplatin was used as positive control.

Table 2. ^{13}C NMR data of 1–3 (1, 3 in $\text{Me}_2\text{CO}-d_6$; 2 in CDCl_3)

No.	1	2	3
	δ_{C} mult	δ_{C} mult	δ_{C} mult
1	65.2, t	64.1, t	63.9, t
2	135.2, s	130.2, s	140.3, s
3	132.8, d	137.9, d	131.0, d
4	77.6, s	77.6, s	77.7, s
5	79.4, d	77.9, d	79.4, d
6	33.4, t	32.2, t	34.7, t
7	39.4, s	38.5, s	39.3, s
8	22.0, q	21.7, q	20.4, q
9	45.3, d	44.0, d	52.4, d
10	41.9, t	41.5, t	220.6, s
11	38.4, s	37.9, s	44.6, s
12	48.4, t	47.4, t	41.9, t
13	39.9, d	39.2, d	35.0, d
14	32.3, q	31.8, q	28.2, q
15	32.2, q	31.7, q	27.4, q
1'	105.9, s	104.6, s	105.9, s
2'	166.3, s	165.7, s	166.4, s
3'	99.5, d	98.8, d	99.5, d
4'	165.0, s	164.2, s	165.5, s
5'	111.6, d	111.3, d	111.5, d
6'	144.1, s	143.0, s	144.3, s
7'	24.2, q	24.2, q	24.3, q
8'	171.8, s	171.3, s	171.8, s
OCH ₃	55.7, q	55.3, q	55.8, q
CHO		160.8, d	

Experimental Section

General Experimental Procedures. Optical rotations (OR) were recorded on a Jasco P-1020 digital polarimeter. UV and the IR spectra were obtained on a Shimadzu UV2401PC and a Bruker Tensor 27 FT-IR spectrometer with KBr pellets. Nuclear Magnetic Resonance (NMR) spectra were obtained on a BRUKER AM-400 and a BRUKER DRX-500 MHz spectrometer with tetramethylsilane (TMS) as an internal standard at room temperature. High-resolution (HR) ESIMS were recorded on an API QSTAR Pulsar spectrometer. Silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., China) and Sephadex LH-20 (Amersham Biosciences, Sweden) were used for open column chromatography (CC). Preparative HPLC was performed on an Agilent 1100 liquid chromatography system equipped with a Zorbax SB-C18 column (9.4 mm \times 150 mm). Fractions were monitored by TLC. Spots were visualized by heating silica gel plates immersed in Vanillin- H_2SO_4 in ethanol.

Fungal Material and Cultivation Conditions. The fungus *Arimillaria* sp. was collected from Wuding, in Yunnan Province, China, in 2005. The culture medium consisted of glucose (5%), peptone from porcine meat (0.15%), yeast powder (0.5%), KH_2PO_4 (0.5%) and MgSO_4 . Fermentation was carried out on a shaker at 160RPM for 25 days.

Extraction and Isolation. The culture broth (21 L) was filtered, and the filtrate was extracted three times with ethyl acetate, while the mycelium was extracted three times with

CHCl_3 -MeOH (1:1). The EtOAc layer together with the mycelium extraction was concentrated under reduced pressure to give a crude extract (20 g), and this residue was chromatographed on silica gel (200–300 mesh) column eluted with a gradient of CHCl_3 -MeOH (1:0 \rightarrow 0:1) to obtain six fractions (1–6). Fraction 4 was subjected to a silica gel CC (petroleum ether-EtOAc, 6:1) to give 4 sub fractions (4a–4d). Subfraction 4c was further purified by Sephadex LH-20 column (eluted with acetone) to give **2** (6 mg) while 4d was subjected to Sephadex LH-20 column (eluted with CHCl_3 -MeOH, 1:1) followed by semipreparative HPLC ($\text{MeCN}-\text{H}_2\text{O}$, eluting from 55:45 to 100:0 for 40 min with a flow rate of 10 mL/min) to yield **1** (14 mg) and **3** (11 mg).

10-Dehydroxy-melleoliede B (1): colorless oil; $[\alpha]_{\text{D}}^{15} + 41.4$ (c 1.08, MeOH); UV (MeOH) λ_{max} (log ϵ) 302 (3.73), 264 (4.13), 215 (4.35) nm; IR (KBr) ν_{max} 3430, 2949, 2864, 1646, 1617, 1578, 1444, 1257, 1160 cm^{-1} ; ^1H (400 MHz) and ^{13}C NMR (100 MHz) data ($\text{Me}_2\text{CO}-d_6$), see Tables 1 and 2; negative ion HRESIMS m/z 415.2118 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_{24}\text{H}_{31}\text{O}_6$, 415.2120).

1-O-Formyl-10-dehydroxy-melleoliede B (2): colorless oil; $[\alpha]_{\text{D}}^{15} + 29.4$ (c 0.29, MeOH); UV (MeOH) λ_{max} (log ϵ) 302 (3.72), 264 (4.12), 215 (4.35) nm; IR (KBr) ν_{max} 3432, 2920, 1629 cm^{-1} ; ^1H (400 MHz) and ^{13}C NMR (125 MHz) data (CDCl_3), see Tables 1 and 2; negative ion HRESIMS m/z 443.2070 $[\text{M} - \text{H}]^-$, calcd 443.2069).

10-Oxo-melleoliede B (3): colorless oil; $[\alpha]_{\text{D}}^{15} - 42.5$ (c 0.28, MeOH); UV (MeOH) λ_{max} (log ϵ) 302 (3.70), 265 (4.10), 215 (4.33) nm; IR (KBr) ν_{max} 3431, 2928, 1730, 1642, 1618, 1579, 1256, 1160 cm^{-1} ; ^1H (400 MHz) and ^{13}C NMR (100 MHz) data ($\text{Me}_2\text{CO}-d_6$), see Tables 1 and 2; negative ion HRESIMS m/z 429.1917 $[\text{M} - \text{H}]^-$, calcd 429.1913).

Cytotoxicity Assay. Five human cancer cell lines, breast cancer MCF-7, hepatocellular carcinoma SMMC-7721, human myeloid leukemia HL-60, colon cancer SW480, and lung cancer A-549 cells, were used in the cytotoxic assay. All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA) in 5% CO_2 at 37 $^\circ\text{C}$. The cytotoxicity assay was performed according to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method in 96-well microplates.⁵ Briefly, 100 μL adherent cells were seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition with initial density of 1×10^5 cells/mL. Each tumor cell line was exposed to the test compound dissolved in DMSO at concentrations of 0.0625, 0.32, 1.6, 8, and 40 μmol in triplicates for 48 h, with cisplatin (Sigma, USA) and vinorelbine (National Institute for the Control of Pharmaceutical and Biological Products, China) as positive controls. After compound treatment, cell viability was detected and a cell growth curve was graphed. IC_{50} values were calculated by Reed and Muench's method.⁶

Electronic Supplementary Material

Supplementary material is available in the online version of this article at <http://dx.doi.org/10.1007/s13659-012-0077-1> and is accessible for authorized users.

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